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אני, (שם המבקש, מענו – ולגבי גוף מאוגד – מקום התאגדותו)
I (Name and address of applicant, and, in case of a body corporate, place of incorporation)

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ANTIBODIES AND ANTAGONISTS TO ANTIMICROBIAL PEPTIDES FOR THE PURPOSE OF TREATING, INHIBTING AND PREVENTING AUTOIMMUNE DISEASES

hereby apply for a patent to be granted to me in respect thereof.

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* בקשת חלוקה - Application for Division	* בקשת פטנט מוסף Application for Patent of Addition	* דרישת דין קדימה Priority Claim		
* מבקשת פטנט from Application	* לבקשה / לפטנט To Patent/Appl.	מספר/סימן Number/Mark	תאריך Date	מדינת האיגוד Convention Country
No. מס'	No. מס'			
Dated מיום	Dated מיום			
* יפוי כח: כללי/מיוחד – רצוף בזה / עוד יוגש P.O.A.: general / specific - attached / to be filed later -				
Has been filed in case הוגש בענין				
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ANTIBODIES AND ANTAGONISTS TO ANTIMICROBIAL PEPTIDES FOR THE PURPOSE OF TREATING, INHIBITING AND PREVENTING AUTOIMMUNE DISEASES

Yitzchak Hillman

ABSTRACT

The present invention discloses and entails a procedure for producing anti-peptide monoclonal antibody and antagonists raised against antimicrobial peptides. A particular peptide antibody is raised using immunization of mice with glutaraldehyde-cross-linked synthetic peptides. Monoclonal antibodies are obtained using conventional hybridoma technology and are then screened for blocking antibodies by evaluating the blocking activity of the antibodies obtained. Testing the blocking antibodies effects is done on psoriasis lesions of animal models for psoriasis.

References:

Journal of Investigative Dermatology 119: 384-391, 2002
October 10 issue of the *New England Journal of Medicine*
British journal of Dermatology 2002; 147 : 1127-1134
J Invest Dermatol 2001 117; 91-97).
J Pathol 2002 Nov 198:369-77
Nat Immunol 2002 Jun 3:583-90)
Dig Dis Sci 2002 Jun 47:1349-55

FIELD OF THE INVENTION

The present invention generally relates to a method of producing medication. More specifically, it relates to modulating and preventing as well as treating immune responses such as inflammation and autoimmune conditions such as for example psoriasis and arthritis amongst others by administering an effective amount of antibody and/or other antagonists or blocking agents to antimicrobial peptides.

BACKGROUND OF THE INVENTION

PSORIASIS

Psoriasis has been established as a T-cell mediated autoimmune disease with innate immunity paying a key role. Psoriasis is a result of a cutaneous defect that is triggered by an autoimmune activation **(Journal of Investigative Dermatology 119: 384-391, 2002).** Histologically, Psoriasis is characterized by epidermal hypeprolifiration with abnormal differentiation and infiltration of the epidermis and dermis by neutrophils, lymphcytes, macrophages and mast cells.

Up until today, novel systemic interventions have been developed to treat psoriasis. These include mainly T-cell targeted therapies, monoclonal antibody against chemokine tumor necrosis factor and Cytokine targeted therapies.

Other topical treatments include cell proliferation regulators such as retinoid - vitamin A - analog which modulates or changes the cellular differentiation of the epidermis, corticosteroid creams and ointment and synthetic vitamin D3. These topical treatments are aimed to regulate only the end result (inflammation reactivity of the epidermis) they do not prevent the initial process from occurring.
In contrast, this patent aims to use local, topical and/or systemic treatment aimed at preventing and blocking the root cause of the disease, a procedure never previously provided for patients nor discussed in previous medical journals or papers.

Researchers at National Jewish Medical and Research Center report in the October 10 issue of the *New England Journal of Medicine* evaluated the levels of two antimicrobial peptides, known as LL-37 and HBD-2 and described how microscopic examination of skin samples showed significant amounts of the peptides in the skin of psoriasis patients, but none to minor amounts in skin from atopic dermatitis patients, and none in the skin of healthy controls. Additional analysis indicated that most psoriasis patients had at least 10 times as much of the peptides in their skin as did atopic dermatitis patients.

Reverse transcription-polymerase chain reaction as well as Immunohistochemistry and confocal imaging of skin biopsies fro 1-day old babies shows presence of peptide antibiotics indicating effective innate immune protection prior to birth. (British journal of Dermatology 2002; 147 : 1127-1134). The presence of these peptides in the skin forms a barrier for innate host protection against microbial pathogenesis. Peptide induced Vernix Caseosa in the newly born eventually disappears before full thymus development thereby preventing a joint innate immunity-T cell mediated immune response.

Cutaneous injury (a trigger for psoriasis) induces the release of cathelicidin anti-microbial peptide active against group A streptacoccus **(J Invest Dermatol 2001 117; 91-97).**
Human Beta Defensin-2 (HBD-2).

ARTHRITIS

Antimicrobial peptides are expressed and produced in healthy and inflamed human synovial membranes. Deposition of the antimicrobial peptides lysozyme, lactoferrin, secretory phospholipase A(2) (sPA(2)), matrilysin (MMP7), human neutrophil alpha-defensins 1-3 (HNP 1-3), human beta-defensin 1 (HBD-1), and human beta-defensin 2 (HBD-2) was determined by immunohistochemistry. Expression of mRNA for the antimicrobial peptides bactericidal permeability-increasing protein (BPI), heparin binding protein (CAP37), human cationic antimicrobial protein (LL37), human alpha-defensin 5 (HD5), human alpha-defensin 6 (HD6), HBD-1, HBD-2, and human beta-defensin 3 (HBD-3) was analysed by reverse transcription polymerase chain reaction (RT-PCR). RT-PCR revealed CAP37 and HBD-1 mRNA in samples of healthy synovial membrane. Additionally, HBD-3 and/or LL37 mRNA was detected in synovial membrane samples from patients with pyogenic arthritis (PA), osteoarthritis (OA) or rheumatoid arthritis (RA).
Immunohistochemistry identified lysozyme, lactoferrin, sPA(2), and MMP7 in type A synoviocytes of all samples. HBD-1 was only present in type B synoviocytes of some of the samples. Immunoreactive HBD-2 peptide was only visible in some inflamed samples. HNP1-3 was detected in both healthy and inflamed synovial membranes. The data suggest that human synovial membranes produce a broad spectrum of antimicrobial peptides. Under inflammatory

conditions, the expression pattern changes, with induction of HBD-3 in PA (LL37 in RA; HBD-3 and LL37 in OA) as well as down-regulation of HBD-1 **(J Pathol 2002 Nov 198:369-77).**

MULTIPLE SCLEROSIS

Defensins and lactoferrins exist in CSF. These peptides have antimicrobial expression in some diseases like pneumonia and meningitis which may trigger a pathway. It seems that pathways to MS are similar to Rheumatoid Arthritis where there, antimicrobial peptides reside in the synovial fluid surrounding the joint.

CROHN's DISEASE

Paneth cells (a specific type of cell in the intestine) are required to help promote normal vessel formation in cooperation with bacteria – mice absent Paneth cells were incapable of appropriate blood vessel formation. Of note, colonization by one particular type of bacteria commonly found in normal mouse and human intestine, called Bacteroides thetaiotaomicron, or B. thetaiotaomicron, stimulated blood vessel development as efficiently as implantation of a whole microbial society. The conclusion, B. thetaiotaomicron and Paneth cells work together to stimulate postnatal blood vessel formation.

The antimicrobial peptide human alpha-defensin 5 (HD5) is expressed in Paneth cells, secretory epithelial cells in the small intestine **(Nat Immunol 2002 Jun 3:583-90)**

Human alpha-defensins contribute to local intestinal host defense as part of innate immunity and may be of major relevance in microbial infection and chronic inflammatory bowel disease **(Dig Dis Sci 2002 Jun 47:1349-55)**

SUMMARY OF THE INVENTION

Antibodies:
Monoclonal antibodies toward LL-37 or the defensins proteins will be generated by immunization of mice with glutaraldehyde-cross-linked synthetic peptides. Monoclonal antibodies will be obtained using conventional hybridoma technology.

Accordingly to this technology, A hybridoma can be produced by injecting the specific antigen (anti-microbial peptide) into a mouse, collecting antibody-producing cells from the mouse's spleen, and fusing them with long-lived cancerous immune cells. Individual hybridoma cells are cloned and tested to find those that produce the desired antibody. Their many identical daughter clones will secrete, over a long period of time, the made-to-order "monoclonal" antibody.

Screening for blocking antibodies
To evaluate the blocking activity of the antibodies obtained, their ability to block the anti-microbial activity of cathelicidins or β defensins will be tested. This will be tested by a colony-forming unit assay performed with Staphylococcus aureus (isolated from clinical sample), GAS (NZ131), and enteroinvasive Escherichia coli O29 as described (Porter et al, 1997). Before analysis, the concentration of the bacteria in culture will be determined by plating different bacterial dilutions. Cells were washed twice with 10 mm sodium phosphate buffer (20 mm $NaH_2PO_4.H2O$, 20 mm $Na_2HPO_4.7H_2O$) and diluted to a concentration of $2x10^6$ cells per ml (S. aureus, GAS) or $2x10^5$ cells per ml (E. coli) in phosphate buffer. S. aureus and E. coli will be incubated for 4 h at 37°C with various concentrations of LL-37 or β defensins peptides in the presence of various concentrations of antibodies to be examined in 50 µl of buffers using wells of a 96 well round bottom tissue culture plate (Costar 3799, Corning inc., NY). GAS will be incubated for 1 h due to the poor ability of GAS to grow in these buffers. After incubation, the cells will be diluted from 10x to 10^5x , and each of 20 µl of those solutions will be plated in triplicate on tryptic soy broth (for S. aureus) and Todd Hewitt broth (for GAS and E. coli), then the mean number of colonies will be determined. The number of cfu per ml will be calculated, and the blocking activity of the examined antibodies to block the bactericidal activities of the peptides will be calculated as follows: (cell survival after peptide incubation)/(cell survival after incubation without peptide)x100, which represented the percentage of cells that are alive (% live), compared to (cell survival after peptide+antibody incubation)/(cell survival after incubation antibody without peptide)x100.

Testing the blocking antibodies effects on psoriasis lesions - Psoriasis animal models:

The identified blocking antibodies will be further tested for their ability to affect psoriatic lesions by treating animal models for psoriasis. Several models are available.
Human Psoriatic Skin–SCID Mouse Transplant Model: Transplantation of human skin onto immunocompromised mice (either congenitally athymic [nude] mice or severe combined immunodeficiency [SCID] mice) provides one of the an approach to the study of psoriasis.
SCID mice (CB-17 strain; Taconic Farms Inc., Germantown, New York) will be used as tissue recipients. Keratomed tissue samples will be obtained from normal or psoriatic volunteer and cut into 1 x 1 cm sections. Two to four mice will be transplanted bilaterally with each human

skin sample, depending on tissue availability. After mice will be anesthetized (sodium pentobarbital; 1.8 mg per 25 gm body weight, i.p.), the dorsal region of each mouse will be shaved bilaterally. Mouse skin will be surgically removed to size, and replaced with the human tissue. The transplanted tissue will be secured to the back of the mouse with absorbable sutures (4-0 Dexon "S"; Davis-Geck, Manati, Puerto Rico). The transplants will be further bandaged with Xeroform petrolatum dressing for 5 days. The animals will be maintained in a pathogen-free environment throughout the preparation and treatment phases. Antibody screening will be initiated 3 to 5 weeks after transplantation.

Flasky skin (fsn) mouse model: Another model that will be tested is a murine model that express a psoriasifrom phenotype i.e., the flasky skin (*fsn*) mutation. Breeding pairs of CBy.A fsn/J mice (The Jackson Laboratory, Bar Harbor, ME) will be obtained. As the genetic defect resulting in the flaky skin phenotype is unknown and as homozygous mutant mice are not fertile, the offspring of $CBY^{FSN/fsn}$ mice will be used for all experiments. In the CBy.A background, erythrosquamous skin lesions are readily seen at the age of 5-6 weeks, allowing the separation of fsn/fsn mice from their wild-type or heterozygous littermates. For antibody treatment studies, mice will be used between 12 and 16 weeks of age (littermates in most cases), after it has been established that the phenotype remained stable within this time frame.

Treatment protocols:

Animals will be divided into treatment groups (vehicle plus test reagents) or a nontreatment group (vehicle alone). The monoclonal antibodies will be delivered intraperitoneally in 100 µL of PBS (6 mg/kg of body weight as an initial concentration used. This will be adjusted according to results). The control mice were treated with PBS alone. Treatment was continued daily for 14 days.

Quantitative Evaluation of Epidermal Thickness:

After the treatment phase, mice will be killed and the transplanted human tissue surgically removed and fixed in 3% formalin. After paraffin embedding, one to three 5-µm-thick sections will be cut from each tissue piece, mounted onto microscope slides, and stained with hematoxylin and eosin. The epidermal area will be measured as a function of changes in epidermal thickness per unit length using NIH Image software (National Institutes of Health, Bethesda, Maryland). Specifically, randomly chosen tissue section fields will be visualized by light microscopy at x10 magnification. At this level of magnification, the entire epidermal area of each tissue section is "captured" in equal segments (three to four segments across a typical tissue section), and the area of each segment can be quantified using the NIH Image analysis program. Multiple areas from bilateral transplants on two to four mice per treatment group for each donor will be quantified in this way, to provide 100 or more measurements. The mean epidermal area will be determined from these values. For the Human Psoriatic Skin–SCID Mouse Transplant Model an additional control value will be set; Before transplantation, a small piece of tissue from each donor will be fixed in 3% buffered formalin and used for zero-time assessment of epidermal thickness.

Histology and Immunohistochemical Assessment

Several other histologic characteristics of psoriasis will be followed to evaluate the effectivness of treatment. This including epidermal hyperplasia, increased rete peg formation, and dermal and/or intra-epidermal infiltration with lymphocytes and neutrophils. For this purpose 5-mm-thick sections will be obtained from each tissue piece, stained with hematoxylin and eosin, and evaluated microscopically.

Statistical Analysis:

Statistical significance will be assessed by the paired two-tailed Student's t-test, and $P < 0{\cdot}05$ will be considered significant. In addition, measurements of epidermal thickness for each group will be analyzed by ANOVA and comparisons between paired groups. The analysis accounts for the correlation between pre-treatment values and post-treatment values for each individual tissue, using a mixed model approach.

POLYTHERAPY

Polytherapy using antimicrobial peptide and psoriatic pathway inhibitory components are included in this patent as complementary assisting use (by addition in saline or lipid solution) by any one or combination or all of the following:
Peptide inhibitors such as protease inhibitors, the serpine serine proteinase inhibitory components (alpha-1 PI) and alpha -1 antichyrmopsin. Am J respin. Cell Mol. Biol. 12: 351-357, BAPTA-AM (an intracellular Ca(2+) chelating agent), pertussis toxin and U-73122 (a phospholipase C inhibitor) (Eur J Immunol 2001 Apr 31:1066-75), T-cell targeted therapies, monoclonal antibody against chemokine tumor necrosis factor and Cytokene targeted therapies, fibroblast growth factor inhibitors, topical treatments include cell proliferation regulators such as retinoid - vitamin A - analog which modulates or changes the cellular differentiation of the epidermis, Tazarotene, methotrexate, acitretin, bexarotene, ploralem, etretinate, corticosteroid creams and ointment and synthetic vitamin D3.

MIMICS

The polymer is prepared by cross-linking a monomer around a "template molecule" (the antimicrobial peptide). This template molecule is removed after the polymerisation of the monomer and its size, shape and chemical functions are recorded in the polymer. The sites of the removed template molecule are named "imprint sites". These sites allow the recognition of the template molecule or close structural molecules

Molecularly imprinted polymers can serve as artificial binding mimics as do natural antibodies.

Another method is enclosed in (US Patent 5,770,380), Synthetic antibody mimics--multiple peptide loops attached to a molecular scaffold.

LIST OF ANTIMICROBIAL PEPTIDES

In this patent, antagonists or blockers for antimicrobial peptides includes blockers or antagonists for all or any one or any combination of all antimicrobial peptides including the following:

Alpha-defensins, beta-defensins1 to 6, **Science 2002 Nov 298:995-1000** histones H2A and

H2B (**J Immunol 2002 Mar 168:2356-64**), glycosaminoglycans (**J Investig Dermatol Symp Proc 2000 Dec 5:55-60**) cathelicidins, defensins, LL-37, hCAP18 (protein), β defensins , α defensins **Dig Dis Sci 2002 Jun 47:1349-55**, hepcidins **Eur J Biochem 2002 Apr 269:2232-7**, ubiquicidin (UBI) **J Nucl Med 2001 May 42:788-94**, human lactoferrin (hLF), lysozyme, lactoferrin, secretory phospholipase A(2) (sPA(2)), matrilysin (MMP7), human neutrophil alpha-defensins 1-3 (HNP 1-3), human beta-defensin 1 (HBD-1), and human beta-defensin 2 (HBD-2) ,heparin binding protein (CAP37), human cationic antimicrobial protein (LL37), human alpha-defensin 5 (HD5), human alpha-defensin 6 (HD6), HBD-1, HBD-2, and human beta-defensin 3 (HBD-3) **J Pathol 2002 Nov 198:369-77**, human DCD-1 **J Immunol Methods 2002 Dec 270:53,** HE2alpha and HE2beta1, human HE2-gene derived transcripts, HE2beta1 **Biol Reprod 2002 Sep 67:804-13**, HE2alpha C-terminal fragments, Human β defensins 1 , β defensins 2, β defensins 3, β defensins 4, HD-5, HD-6, homolog HtpG, Bactericidal/permeability-increasing protein [BPI] **Mol Microbiol 1995 Aug 17:523-31,** Dermicidin **Nat Immunol 2001 Dec 2:1133-7**,

APPLICATION METHODS

Local injection in saline solution in cases of for example arthritis.
Topical application in lipid or saline solution or in cream on the skin of psoriasis legion.
Inhaler in solution in Cystic fibrosis and for asthma.

Cream solutions can include any lipids or organic alcohols or chemicals including for example benzyl alcohol, macrogol, hexylene glycol, carbomer, ascorbic acid, butyl hydroxyainisole, butyl hydroxytoluene, disodium edentate, water, trometamol, poxoamer.

AUTOIMMUNE DISEASED THAT ARE TRIGGERED BY ANTIMICROBIAL PEPTIDES

a. crohn's disease
b. psoriasis
c. dermatitis
d. arthritis and rheumatoid arthritis
e. atherosclerosis
f. asthma
g. cystic fibrosis
h. multiple sclerosis
i. diabetes
j. lupus
k. scleroderma
l. thyroid inflammatory diseases
m. celiac disease
n. fatigue syndromes
o. eating disorders
p. graves disease
q. reiters syndrome

r. myasthenia gravis
s. dermatomyosis
t. addison's disease
u. pernicious anemia
v. Guillain-Barre' syndrome
w. fibromyalgia
x. goodspature
y. atopic allergy
z. celiac
aa. all other autoimmune diseases listed in medical dictionaries as "autoimmune diseases" and that are triggered by antimicrobial peptides.

What is claimed:

1. A method for treating and preventing disease

2. a method in claim 1 in which the disease is an inflammation or autoimmune diseases

3. a method in claim 2 in which the procedure uses blocking agents in order to treat and to inhibit the cause of inflammation and its pathways

4. the method in claim 3 in which the blocking agent is a monoclonal antibody, antibody fragment, mixture or derivative thereof, antagonist or mimics that bind to peptides, polypeptides, antimicrobial peptides, and proteins and inhibits their activity

5. a monoclonal antibody, antibody fragment, mixture or derivative thereof, antagonist, or a mimic which binds to peptides and inhibits its activity and which is produced by the method in claim 4

6. a cell which expresses a monoclonal antibody, antibody fragment, mixture or derivative thereof of type as in claim 5

7. a cell as claimed in claim 6, which is from a hybridoma cell line

8. a hybridoma according to claim 7, wherein the hybridoma is a mouse hybridoma

9. a pharmaceutical preparation comprising a monoclonal antibody, antibody fragment, mixture or derivative thereof as claimed in claim 3

10. compositions for treating autoimmunity in a patient undergoing therapy comprising a monoclonal antibody, antibody fragment, mixture or derivative thereof as claimed in claim 5

11. a method for screening for the blocking monoclonal antibodies in claim 5

12. the method in claim 11 using survival rates in cultures of microbes or bacteria with the antimicrobial peptides and the antibodies or antagonists in claim 5.

13. a method for testing the effectiveness of the antibody or antagonist in claim 5 on psoriasis lesions Psoriasis animal models

14. the method in claim 13 using Quantitative Evaluation of Epidermal Thickness

15. the method in claim 14 using statistical analysis and ANOVA.

16. An autoimmune disease in claim 2 that includes amongst others the following diseases:

a. crohn's disease
b. psoriasis
c. dermatitis
d. arthritis and rheumatoid arthritis
e. atherosclerosis
f. asthma
g. cystic fibrosis
h. multiple sclerosis
i. diabetes
j. lupus
k. sclerodema
l. thyroid inflammatory diseases
m. celiac disease
n. fatigue syndromes
o. eating disorders
p. graves disease
q. reiters syndrome
r. myasthenia gravis
s. dermatomyosis
t. adison's disease
u. pernicious anemia
v. guillen bar
w. fibromyalgia
x. goodspature
y. atopic allergy
z. celiac
aa. all other autoimmune diseases listed in medical dictionaries as "autoimmune diseases" and that are triggered by antimicrobial peptides.

17. an antimicrobial peptide referred to in claim 4 that is one or any combination of the following:

a. cathelicidins
b. defensins
c. LL-37
d. hCAP18 (protein)
e. β defensins
f. α defensins
g. hepcidins
h. ubiquicidin (UBI)
i. human lactoferrin (hLF)

j. human DCD-1
k. HE2alpha and HE2beta1
l. human HE2-gene derived transcripts
m. HE2beta1
n. and HE2alpha C-terminal fragments
o, Human β defensins 1 , β defensins 2, β defensins 3, β defensins 4, HD-5, HD-6
p. eNAP-1, HtpG
q. lactoferrin
r. histones, H2A, H2B
s. dermicidin